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BIODIVERSITY OF THE SYMBIOTIC SYSTEMS FORMED BY NODULE BACTERIA *Rhizobium leguminosarum* WITH THE LEGUMINOUS PLANTS OF GALEGOID COMPLEX

O.P. ONISCHUK[1], O.N. KURCHAK[1], A.K. KIMEKLIS[1], T.S. AKSENOVA[1], E.E. ANDRONOV[1, 2], N.A. PROVOROV[1] ✉

[1]*All-Russian Research Institute for Agricultural Microbiology,* 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail provorovnik@yandex.ru (✉ corresponding author), olony@yandex.ru, okurchak@yahoo.com, kimeklis@gmail.com, tsaksenova@mail.ru, eeandr@gmail.com;
[2]*Saint-Petersburg State University,* 7-9, Universitetskaya nab., St. Petersburg, 199034 Russia, e-mail eeandr@gmail.com
ORCID:
Onishchuk O.P. orcid.org/0000-0002-5378-7826
Kurchak O.N. orcid.org/0000-0003-3555-7426
Kimeklis A.K. orcid.org/0000-0003-0348-7021
Aksenova T.S. orcid.org/0000-0002-7294-8410
Andronov E.E. orcid.org/0000-0002-5204-262X
Provorov N.A. orcid.org/0000-0001-9091-9384
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Abstract

Nodule bacteria of the species *Rhizobium leguminosarum* are differentiated into two biovars (bv.) that form N_2-fixing symbioses with leguminous plants of the galegoid complex, tribes *Fabeae* (genera *Lathyrus, Lens, Pisum, Vavilovia, Vicia*, symbiont — *R. leguminosarum* bv. *viciae*) and *Trifolieae* (genus *Trifolium*, symbiont — *R. leguminosarum* bv. *trifolii*) (J. Sprent et al., 2017). It was previously assumed that cross-inoculation between these biovars is impossible or rare, while data on the control of host specificity of *R. leguminosarum* were limited by interactions between pea (*P. sativum*) lines with different alleles of *Sym2* gene and bv. *viciae* strains that differ in the presence of *nod*X gene (T.A. Lie, 1978). The aim of our work was to analyze the variability of *R. leguminosarum* bv. *viciae* strains from ancestral (A) and evolutionarily advanced (D) genomic groups in terms of host specificity and N_2-fixing activity, aimed at the functional characterization of ancestral genome elements, which were previously identified by the comparative genetic analysis of strains isolated from representatives of the *Fabeae* tribe that differ in phylogenetic affiliation. In accordance with the previously proposed genotyping technique, strains were assigned to group A if they contained the *nod*X and *fix*W genes, did not contain a chromosomal copy of the *fix*NOPQ operon, and the *nod*T gene was outside the *nod* cluster. In the absence of at least one of these features, the strains were assigned to group D (E. Chirak et al., 2019). Group A strains were isolated from the relict legume *Vavilovia formosa* and from wild-growing Afghan lines of *P. sativum*, group D strains were isolated from cultivated European lines of *P. sativum*, from *Vicia sativa* and *V. alpestris*. In experiments on the analysis of cross-inoculation of two *R. leguminosarum* biovars we used bv. *viciae* strains isolated from nodules of *Vavilovia formosa, Vicia sativa, V. subrotunda*, European lines of *Pisum sativum*, Afghan lines of *P. sativum*, as well as bv. *trifolii* strains from clover (*Trifolium pratense, T. ambiguum, T. montanum*) nodules. In microvegetative experiments, plants inoculated with rhizobia were grown under gnotobiotic conditions on vermiculite. N_2-fixing activity was determined using the acetylene method based on the use of C_2H_2 as a substrate for nitrogenase. Based on the results obtained, the following symbiotic phenotypes were identified: Fix^+ — N_2-fixing (large, pink) nodules; Fix^- — non-fixing N_2 (small, white, but morphologically normal) nodules; $Fix^{+/-}$ — nodules not fixing N_2, but similar to Fix^+ nodules (large, pink); Ndv^- — non-fixing N_2, tumor-like nodules; Nod^- — nodules were absent. It turned out that 9 out of 11 strains of the ancestral group formed on clover nodules of Fix^- phenotype, and 2 strains formed nodules of Ndv^- phenotype. Among 8 strains of the evolutionarily advanced group, the Fix^- and Ndv^- phenotypes were detected in 4 and 2 strains, respectively, and 2 strains did not form nodules on clover (Nod^-), indicating a narrowing of the host specificity of rhizobia during coevolution of bv. *viciae* with host plants. Therefore, we have shown for the first time that during the transition of bv. *viciae* strains to symbiosis with evolutionarily young representatives of the tribe *Fabeae* (transition from the A- to the D-group), bacteria lose the ability to form symbiosis with a heterologous host (*Trifolium*). Among 6

strains of clover rhizobia, 4 strains showed the ability to inoculate vetch forming Fix⁻ nodules. In experiments to control the absence of contamination, DNA was isolated from nodules using the NucleoSpin™ Soil (Macherey-Nagel GmbH & Co. KG, Germany), the *nod*A gene fragment was amplified using universal primers for *R. leguminosarum* biovars (ndARL302_F YTDGGMATCGCHCACT/ndARL518_R RDACGAGBACRTCTTCRGT). The data obtained showed that under the conditions of sterile microvegetation experiments there is no contamination and the majority of strains are able for cross-inoculation: bv. *viciae* strains form nodules on clover and bv. *trifolii* form nodules on vetch. However, this ability is limited by the formation of non-fixing N_2 nodules in heterologous hosts, including morphologically abnormal (tumor-like) nodules. The study of symbioses formed by 9 species of leguminous plants of tribe *Fabeae* (*Pisum sativum, Vicia sativa, V. villosa, V. alpestris, Vavilovia formosa, Lens culinaris, L. nigricans, Lathyrus pratensis, L. sylvestris*) with 6 *R. leguminosarum* bv. *viciae* strains, demonstrated a pronounced specificity of N_2-fixing symbiosis formation, which depends mostly on the bacteria origin. Strains isolated from the same legume species (*V. formosa* or *P. sativum*) are more similar in host specificity than strains from different hosts. A hypothetical scheme of *R. leguminosarum* evolution is proposed, according to which: a) divergent evolution of bv. *viciae* is determined by host plant speciation in tribe *Fabeae*; b) the closest relative of a common ancestor of *R. leguminosarum* is represented by bv. *trifolii*, which display an evolutionary primitive *sym* gene organization and possibly originated from the ancestral bv. *viciae* strains that changed their host specificity. The rhizobia isolated from *V. formosa* may be considered as the close relatives of these ancestral strains since they exceed the pea and vetch isolates in the ability to form morphologically normal nodules with the heterologous host, clover. The data obtained show the possibility of constructing rhizobia strains with an increased symbiotic activity by editing the ancestral components of their genomes.

Keywords: nodule bacteria, leguminous plants, symbiotic N_2-fixation, host specificity, biodiversity, *Rhizobium leguminosarum*, genomic groups, evolution of symbiosis

Symbioses of leguminous plants with N_2-fixing nodule bacteria (rhizobia) are very diverse in their structural and functional organization and the specificity of the interaction of partners [1]. The most studied symbioses are those formed by bacteria of the family. *Rhizobiaceae* (genera *Rhizobium*, *Sinorhizobium*, *Neorhizobium*) with plants of the galegoid complex, including the tribes *Fabeae*, *Galegae* and *Trifolieae*. Rhizobia infect these plants through root hairs where infection threads arise. Infection threads are tubular structures that allow rhizobia to enter intracellular symbiosomes with subsequent transformation into irreversibly differentiated N_2-fixing bacteroids [2].

Symbioses formed by galegoid legumes are highly specific interactions of partners within separated cross-inoculation groups (CIG) [3]. For example, the species *Rhizobium leguminosarum* is divided into two biovars that form different CIGs. Their hosts are plants of the IRLC group of the galegoid complex, whose plastid DNA does not carry the 25-kb inverted repeat found in most galegoid legumes. Plant symbionts of the tribe *Fabeae* (genera *Lathyrus*, *Lens*, *Pisum*, *Vavilovia*, and *Vicia*) are classified as biovar (bv.) *viciae*, and symbionts of the genus *Trifolium* (tribe *Trifolieae*) are classified as bv. *trifolii*. Analysis of the genetic diversity of *R. leguminosarum* strains showed that the biovars *viciae* and *trifolii* differ in the structure of the symbiosis *sym* genes of the accessory part of the genome much more strongly than in the genes of its core part which determine housekeeping functions [4, 5].

We have previously shown [6] that strains of *R. leguminosarum* bv. *viciae* are divided into two genomic groups, the ancestral (A) and evolutionarily advanced (D). The characteristic features of group A strains from the nodules of *Vavilovia formosa*, the putative closest relative of the common ancestor of the tribe *Fabeae* [7], and wild (Afghan) forms of the common pea *P. sativum* are i) the presence of the *nod*X gene for broad host specificity control [8] and the gene *fix*W encoding an enzyme that breaks down disulfide bonds in proteins [9], ii) absence of a chromosomal copy of the f*ix*NOPQ operon, encoding cytochrome oxidase with high affinity for O_2 [10], iii) more than 90 kb plasmid cluster of *sym* genes, and iv) location of the *nod*T gene outside this cluster. Group D strains lack the *nod*X and *fix*W genes, a chromosomal copy of the *fix*NOPQ operon is detected, the *sym* gene

cluster is less than 60 kb in size, and *nod*T is included in the *nod* gene cluster. It is assumed that the transition from the A- to the D-group, which occurred due to the coevolution of rhizobia and their hosts, led to an increase in the adaptability of bacteria to plant-soil systems associated with the activity of symbiosis [11].

This work shows for the first time that during the transition of bv. *viciae* to symbiosis with evolutionarily young representatives of the tribe *Fabeae* (transition from A- to D-group), the bacteria lose the ability to form symbiosis with a heterologous host (*Trifolium*). Under conditions of sterile micro-pot tests, most bv. *viciae* strains which form nodules on clover and bv. *trifolii* strains which form nodules on vetches exhibit the ability to cross-inoculate. However, this ability is limited by the formation of nodules in heterologous hosts that do not fix N_2, including those with abnormal morphology (tumor-like). A hypothetical scheme for the evolution of the species *R. leguminosarum* has been proposed according to which the divergence of bv. *viciae* is determined by the increasing diversity of plants of the tribe *Fabeae*. The bv. *trifolii* is the closest to the common ancestor of this species, it retained the evolutionarily primitive organization of *sym* genes and possibly arose through changes in host specificity in ancestral strains of bv. *viciae*, the closest relatives of which can be considered the symbionts of *V. formosa*.

The purpose of our work was to analyze the variability of *Rhizobium leguminosarum* bv. *viciae* ancestral (A) and evolutionarily advanced (D) genomic groups in terms of host specificity and N_2-fixing activity, aimed at the functional characterization of ancestral elements of the genome, which were previously identified during a comparative genetic analysis of strains isolated from the tribe *Fabeae* representatives that differ in phylogenetic position.

Materials and methods. The experiments used strains of *R. leguminosarum* bv. *viciae* from the collection of the All-Russian Research Institute of Agricultural Microbiology, isolated from the nodules of *Vavilovia formosa* (Steven) Fed. (Vaf10, Vaf12, Vaf13, Vaf35, Vaf25, Vaf01, Vaf46, Vaf96, Vaf108, 1B2, 1G1), *Vicia sativa* L. (Vst35-4, Vst36-3, 1-32), *V. subrotunda* (Maxim.) Czefr. (Vs35-4, Vs36-3, Vs37-3, 2S1, 3S1, 2-1k, 3Vsb, 1Vsd, L1-1, L2-1), European lines of *Pisum sativum* L. (1079, CIAM1026, Wp1, Wp3, Wp40, Wp19, Wp24, Wp60), Afghan lines of *P. sativum* L. (A1, TOM), as well as strains of *R. leguminosarum* bv. *trifolii* from clover nodules (*Trifolium pretens*e L., *T. ambiguum* M. Bieb., *T. montanum* L.) (Tp73-4, Tr11, Tm2, Ta6, Ka1, Ka5). Strains of alfalfa rhizobia *Sinorhizobium meliloti* (AK57, A18) and goat's rue *Neorhizobium galegae* (Gr12/7, Gr1025), which do not form nodules on host plants of the species *R. leguminosarum*, were used as negative controls in cross-inoculation experiments.

Seeds of *Vicia alpestris* Steven (I-0146902), *V. sativa* L. (variety Nikolskaya, K-36638), *V. villosa* Roth. (variety Lugovskaya 2, K-37019), *Lens culinaris* Medikus (variety Pikantnaya, K-3051), *L. nigricans* (M.Bieb.) Godr. (ILWL37), *Lathyrus pratensis* L. (N094275), *L. sylvestris* L. (K 1959), *T. pratense* L. (cultivar Suydinets, K-34600) were obtained from the collection of the Vavilov All-Russian Research Institute of Plant Resources, seeds of *P. sativum* L. (Afghan line NGB2150, European lines Frisson and SGE) derived from the collection of the All-Russian Research Institute for Agricultural Microbiology, seeds of *V. formosa* (Steven) Fed. were collected from wild plants in the Caucasus [12].

In micro-pot experiments, plants inoculated with rhizobia were grown under gnotobiotic conditions on vermiculite with nitrogen-free Krasilnikov-Korenyako medium in 60 ml test tubes (*Trifolium*, *Lathyrus*, *Vicia*) or in 1000 ml glass cylinders (*Lens*, *Pisum*, *Vavilovia*). N_2-fixing activity was determined using the acetylene method with C_2H_2 as a substrate for nitrogenase [13]. Roots with nodules were placed in hermetically sealed 50 ml bottles, into which 2.5 ml of C_2H_2 (5% volume) was introduced and incubated for 1 hour. In this case, the reduction of N_2 is blocked and the formation of C_2H_4 occurs, the amount of which was

determined on a gas chromatograph GC-2010 (Shimadzu, Japan). Based on the results of micro-pot tests, the following symbiotic phenotypes were identified: Fix^+ — N_2-fixing (large, pink) nodules; Fix^- — non-fixing N_2 (small, white, but morphologically normal) nodules; $Fix^{+/-}$ — nodules that do not fix N_2 and are similar in appearance to Fix+ nodules (large, pink); Ndv — non-N_2 fixing, tumor-like nodules; Nod^- — without nodules.

DNA was isolated from nodules using the NucleoSpin™ Soil kit (Macherey-Nagel GmbH & Co. KG, Germany), the *nod*A gene fragment was amplified at 30 s, 95 °C, 30 s, 50 °C, 30 s, 72 °C (35 cycles) using universal primers for *R. leguminosarum* biovars (ndARL302_F YTDGGMATCGCHCACT/ndARL518_R RDACGAGBACRTCTTCRGT). Sequencing of amplicon libraries was carried out using Illumina technology (an Illumina MiSeq instrument, Illumina, Inc., USA) with a MiSeq® ReagentKit v3 (600 cycle) with double-sided reading (2×300 nt). The obtained sequences were processed using Illumina software.

In accordance with the previously proposed genotyping technique [6], strains were assigned to the ancestral genomic group A if they contained the *nod*X and *fix*W genes, did not contain a chromosomal copy of the *fix*NOPQ operon, and the *nod*T gene was located outside the *nod* cluster. In the absence of at least one of these characters, the strains were assigned to the evolutionarily advanced group D.

Statistical processing of the results was carried out using analysis of variance and Student's *t*-test, the values of which (t_{St}) were used to assess the probability of the null hypothesis (P0) about the absence of differences between the studied samples of strains [14]. Strains were considered N_2-fixing (Fix^+) if the amount of C_2H_4 in the experimental sample was significantly higher than in the control without inoculation. To carry out cluster analysis, symbiotic phenotypes were given numerical values (0 — Nod^-, 1 — Fix^-, 2 — $Fix^{+/-}$, 3 — Fix^+), which were used to calculate Euclidean distances between strain phenotypes, to construct a dendrogram using the UPGM method and to statistically support clusters bootstrap using PAST program [15].

Results. Firstly, we analyzed the cross-inoculation of biovars *viciae* and *trifolii* with their host plants (tribe *Fabeae* and genus *Trifolium*). The results of micro-pot tests showed (Table 1, Fig. 1) that among the 33 tested *R. leguminosarum* bv. *viciae* strains isolated from peas, vetch and Vavilovia, 28 strains formed on meadow clover (*T. pratense*) nodules that did not fix N_2 (23 strains formed morphologically normal nodules of the Fix^- phenotype, 5 strains formed tumor-like nodules of the Ndv^- phenotype), and 5 strains did not formed nodules (Nod^- phenotype). It is important to note that Fix^- nodules on clover were formed by 91.6% of strains isolated from Vavilovia, and only 57.1% of strains from vetch and peas (t_{St} = 2.57; P0 < 0.05). In addition, on vetch plants, Fix^+ nodules were formed by only 41.7% of strains isolated from Vavilovia, and 100% of strains from vetch and peas (t_{St} = 2.93; P0 < 0.05), which indicated the specialization of Vavilovia symbionts to their to the host.

To compare the phenotypic variation of the studied strains of *R. leguminosarum* bv. *viciae* with their genomic organization, using the previously proposed method [6], we identified among them the ancestral (A) and evolutionarily advanced (D) groups (see Table 1). It turned out that 9 out of 11 strains of group A exhibited the Fix^- phenotype on clover, and 2 strains exhibited the Ndv^- phenotype. Among the 8 strains of group D, the Fix^- and Ndv^- phenotypes were detected in 4 and 2 strains, respectively, and 2 strains did not form nodules on clover (Nod^- phenotype). Of the 6 strains of clover rhizobia (*R. leguminosarum* bv. *trifolii*) we studied, 4 strains exhibited the Fix^- phenotype on hairy vetch (*V. villosa*), and 2 strains exhibited the Nod^- phenotype (see Table 1). When clover and vetch were inoculated with symbionts of alfalfa (*Sinorhizobium meliloti*) and goat's

rue (*Neorhizobium galegae*), as in controls without inoculation, nodules were absent.

1. Cross-inoculation of *Rhizobium leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* of host plants depending on the strain origin and genomic structure

Plants from which strains were isolated	Number of strains studied (number of strains having the gene *nod*X)	Ratio of genomic groups A:D	Symbiotic phenotypes of the strains on plants	
			Vicia villosa Roth.	*Trifolium pratense* L.
R. leguminosarum bv. *viciae*				
Vavilovia formosa (Steven) Fed.	12 (12)	7:0	5 Fix^+, 7 Fix^-	11 Fix^-, 1 Nod^-
Vicia sativa L.	3 (3)	0:3	3 Fix^+	3 Fix^-
V. subrotunda (Maxim.) Czefr.	10 (8)	2:1	10 Fix^+	6 Fix^-, 2 Ndv^-, 2 Nod^-
Pisum sativum L. (European lines)	6 (0)	0:4	6 Fix^+	3 Fix^-, 1 Ndv^-, 2 Nod^-
P. sativum L. (Afghan lines)	2 (2)	2:0	2 Fix^+	2 Ndv^-
R. leguminosarum bv. *trifolii*				
Trifolium spp.	6 (6)	Not tested	4 Fix^-, 2 Nod^-	6 Fix^+

N o t e. For bv. *viciae* the ratio of strains belonging to the ancestral (A) and evolutionarily advanced (D) genomic groups is indicated. Fix^+ — N_2-fixing (large, pink) nodules, Fix^- — not N_2-fixing (small, white, but morphologically normal) nodules, Ndv^- — not N_2-fixing, tumor-like nodules, Nod^- — no nodules.

Fig. 1. Phenotypes of nodules formed on the roots of *Trifolium pratense* L. by various strains of *Rhizobium leguminosarum*: a — Vaf12 bv. *viciae* (tumor-like nodules that do not fix N_2, Ndv^-), b — Vaf108 bv. *viciae* (morphologically normal nodules that do not fix N_2, Fix^-, c — Ta6 bv. *trifolii* (N_2-fixing nodules, Fix^+).

Genotyping showed that the ability of *R. leguminosarum* biovars to form nodules on plants from heterologous cross-inoculation groups is not associated with the presence of the nodX gene. Indeed, the Fix^- phenotype on clover was demonstrated by *nod*X-containing bv. *viciae* strains isolated from Vavilovia and strains from European pea lines lacking this gene. Despite the contrasting variation of bv. *trifolii* in terms of their ability to form nodules on vetch, they all had the *nod*X gene.

To confirm the phenomenon of cross-inoculation between rhizobia of different biovars, excluding contamination of bv. *viciae* suspensions used to inoculate clover, with "spontaneous" strains of bv. *trifolii*, as well as suspensions of bv. *trifolii* used for vetch inoculation with bv. *viciae*, we isolated total DNA from the formed Fix^- nodules, from which amplicon libraries were prepared for the *nod*A gene, which has clear differences in the compared rhizobial biovars [16]. Deep sequencing of these libraries using over 10 thousand *nod*A gene sequences from Fix^- nodules formed by strains Vaf12 and Vaf108 on clover, as well as strain Tr11 on vetch did not reveal contaminants.

At the second stage of work, strains of *R. leguminosarum* bv. *viciae*, representing different genomic groups, were used to inoculate 9 legume species from all 5 genera of the tribe *Fabeae*. Fix^+ nodules were formed in only 19 of 60 genotypic combinations of partners, indicating the high specificity of the formation of N_2-fixing symbiosis (Table 2). Among 3 broadly specific strains that formed Fix_+ nodules with 5-8 plant species, 2 strains (Vaf-12 and A1) represented genomic group A (*nod*X is present), and 1 strain (1079) represented group D (*nod*X is absent). Strains Vaf-10, Vaf-46 and Vaf-108 included in group A formed nodules that did not fix N_2 with all the legumes studied, but in the original host, Vavilovia,

they were similar to Fix^+ nodules (large size, pink color) and were isolated into a separate phenotype ($Fix^{+/-}$). It is important to note that all plant species studied formed Fix^+ nodules with at least one of the tested rhizobia strains. Thereof, the specificity of the formation of N_2-fixing symbiosis depended more significantly on the bacterial strain than on the plant species.

2. Specificity of interaction between different strains of *Rhizobium leguminosarum* bv. *viciae* and plants of the tribe *Fabeae*

Strain	Host plants										
	Pisum sativum		*Vicia*			*Vavilovia*	*Lens*		*Lathyrus*		total
	Afghan	European	*alpestris*	*sativa*	*villosa*	*formosa*	*culinaris*	*nigricans*	*pratensis*	*sylvestris*	Fix^+
Vaf-10 (A)	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	$Fix^{+/-}$	Fix^-	Fix^-	Fix^-	Fix^-	0
Vaf-12 (A)	Fix^-	Fix^-	Fix^+	Fix^+	Fix^+	$Fix^{+/-}$	Fix^+	Fix^+	Fix^-	Fix^-	5
Vaf-46 (A)	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	0
Vaf-108 (A)	Fix^-	Fix^-	Fix^-	Fix^-	Fix^-	$Fix^{+/-}$	Fix^-	Fix^-	Fix^-	Fix^-	0
A1 (A)	Fix^+	Fix^+	Fix^-	Fix^+	Fix^+	Fix^+	Fix^+	Fix^+	Fix^+	Fix^-	8
1079 (D)	Nod^-	Fix^+	Nod^-	Fix^+	Fix^+	Fix^-	Fix^+	Fix^+	Fix^-	Fix^+	6
Total Fix^+	1	2	1	3	3	1	3	3	1	1	19

N o t e. The belonging of strains to genomic groups is indicated in brackets: A — ancestral, D — evolutionarily advanced. Fix^+ — N_2-fixing (large, pink) nodules, Fix^- — not N_2-fixing (small, white, but morphologically normal) nodules, $Fix^{+/-}$ — not N_2-fixing nodules, similar to Fix^+ nodules (large, pink), Nod^- — no nodules.

To study the relationship between host specificity of *R. leguminosarum* bv. *viciae* and their origin, we analyzed the similarity of these strains in terms of symbiotic phenotypes manifested during interaction with 10 plant forms (see Table 2). It turned out that when comparing strains isolated from the same legume species (Vavilovia or pea), the average number of matches for the Fix^+ phenotype is 6±0.97, and among strains from different legume species it is only 2±0.68 (t_{St} = 3.39; P0 < 0.01). Cluster analysis of the obtained data revealed the phenotypic similarity of the Vavilovia strains Vaf10, Vaf46 and Vaf108, forming a compact cluster with a bootstrap support level of 99%, as well as a cluster with a much lower level of support (50%), uniting strains Vaf12 from Vavilovia, A1 from Afghan peas and 1079 from European peas (Fig. 2).

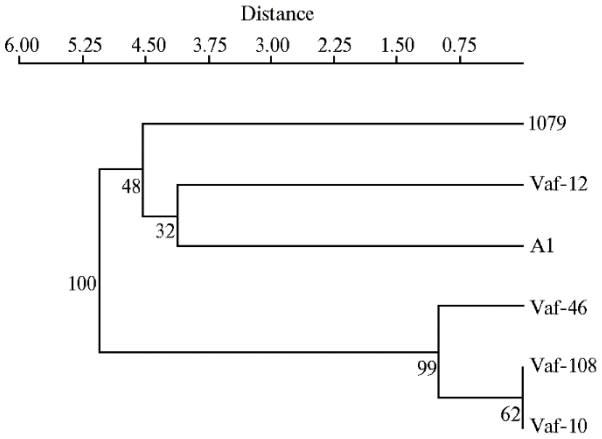

Fig. 2. Cluster analysis of *Rhizobium leguminosarum* bv. *viciae* according to the phenotype manifested in symbiosis with various legume species of the tribe *Fabeae* (based on data in Table 2; Euclidean distances and boot-straps for clusters are indicated).

Symbioses formed by nodule bacteria (rhizobia) and legumes are one of the most developed models for the genetic analysis of interactions between prokaryotes and eukaryotes, as well as for the development of methods for constructing microbial-plant systems for agricultural purposes [17]. Previously proposed approaches to solve this problem include the production of mutants and recombinants with increased N_2-fixing activity, symbiotic efficiency (the ability to increase

the productivity of host plants), as well as the ability to compete with native strains of rhizobia for the formation of nodules in plants.

It has been shown that an increase in the N_2-fixing activity of rhizobia can be achieved through amplification of genes that ensure the formation of the nitrogenase complex and its energy supply [18]. An even more promising approach turned out to be inactivation of negative regulators of symbiosis, including genes that control the conversion of plant-derived C-compounds into storage nutrients poly-beta-hydroxybutyrate [19-21] and glycogen [22], as well as sugar transport [23], high respiration intensity [24] and the formation of surface polysaccharides that protect bacteria from edaphic (osmotic, temperature, water) stresses [25].

Similar approaches can be used to improve the competitiveness of rhizobia. They are based on the amplification of positive regulators of this trait - genes that activate the synthesis of surface polysaccharides [26] and proline dehydrogenase [27], nodule formation [28], as well as on the inactivation of negative regulators of competitiveness, suppressing the expression of nodule formation genes [29], synthesis NADP-dependent dehydrogenase [30] and biofilm formation [31].

Considering that during the evolution of the legume-rhizobium symbiosis its ecological efficiency increased [1], editing the ancestral elements of the rhizobial genome, which are involved in the control of symbiotic properties, seems promising. Previously, to identify these elements in the polytypic (consisting of several biovars differing in host specificity) species *R. leguminosarum*, we used an approach that was based on a comparative genetic analysis of strains isolated from representatives of the tribe *Fabeae* differing in phylogenetic position [11]. Among the strains of *R. leguminosarum* that are promising for identifying ancestral elements of the genome, the most interesting are the symbionts of *Vavilovia formosa*, a relict species of the tribe *Fabeae*, which can be considered the closest relative of its common ancestor [7]. Based on data on the distribution of Vavilovia in the high mountainous regions of the Caucasus and Central Asia, as well as on the peculiarities of the genomic organization of its symbionts, they were determined to be close to the common ancestor of bv. *viciae*, and possibly the entire species *R. leguminosarum* [5, 6].

This article characterizes the variability of *R. leguminosarum* strains that differ in the composition of ancestral elements of the genome, according to their effect on host specificity and N_2-fixing activity, which allows us to assess the possibility of using these elements in biotechnological programs. We studied the host specificity of *R. leguminosarum* by analyzing cross-inoculation of *viciae* and *trifolii* biovars and interactions of bv. *viciae* strains with various species of the tribe *Fabeae*. Previously, the cross-inoculation groups formed by biovars *viciae* and *trifolii* were thought to be completely separate from each other [3]. However, we have shown that most strains of bv. *viciae* form nodules on clover, and most strains of bv. *trifolii* on vetch (see Table 1). Although the cross-inoculation of biovars that we identified was limited to the formation of nodules that did not fix N_2, including those with anomalous morphology, the data obtained indicate an incomplete symbiotic divergence of the biovars *viciae* and *trifolii*, which may be associated with their recent separation from a common ancestor that had broad host specificity.

Previously, based on an analysis of the genomic diversity of the ancestral (A) and evolutionarily advanced (D) groups of strains, we suggested [11] that the transition from group A to group D causes an increase in the adaptive potential of the species *R. leguminosarum* due to the Nod factor transport intensification associated with migration of the *nod*T gene into the *nod* cluster, bacterial respiration in the soil because of acquisition of a chromosomal copy of the *fix*NOPQ operon and deep differentiation of bacteroids determined by the loss of the *fix*W gene.

In this work, it was established that 9 out of 11 strains of group A formed

on clover nodules with normal morphology that did not fix N_2 (Fix^- phenotype). Two strains formed tumor-like nodules, in which, as it was shown by the study of rhizobia mutants defective in synthesis of lipo- and exopolysaccharides [32], the penetration of bacteria into root tissue was impaired (Ndv^- phenotype). In group D, the occurrence of the Fix^- phenotype was lower than among strains of group A. These data indicate that, during the transition from ancestral to evolutionarily developed genome organization, the bv. *viciae* has lost the ability to inoculate clover plants.

Importantly, the ability of the compared biovars of *R. leguminosarum* to form nodules on alien hosts is not associated with the presence of the *nod*X gene, which was previously considered the main determinant of the host specificity of this rhizobial species. This gene, first identified in bv. *viciae* from Afghan pea lines [8], was later found in strains isolated from Vavilovia and some other plants of the tribe *Fabeae* [6], as well as in all bv. *trifolii* strains studied [33]. The submitted data indicate that the loss of the *nod*X gene, characteristic of the D group, apparently was not the direct cause of the diversification of the *R. leguminosarum* species in terms of host specificity, although *nod*X can be considered as a marker of this process.

We assessed the host specificity of bv. *viciae*, which is determined by the formation of N_2-fixing nodules in 9 species of legumes, representing all 5 genera of the tribe *Fabeae* (see Table 2). This specificity is more dependent on bacteria than on plants and is evident when comparing strains isolated from *P. sativum* and its evolutionary predecessor *V. formosa* (see Fig. 2).

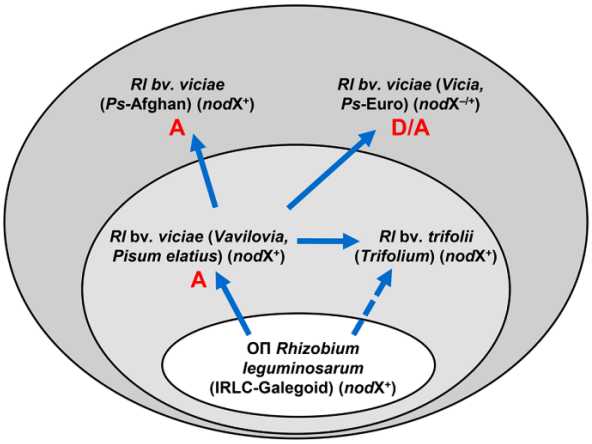

Fig. 3. Divergent evolution of the species *Rhizobium leguminosarum* (*Rl*), which led to the formation of biovars *viciae* and *trifolii*. *Vavilovia* is the closest relative of the common ancestor of the legume tribe *Fabeae*, the hosts of *R. leguminosarum* bv. *viciae*. *Pisum elatius* Bieb. is a wild pea species whose range overlaps with the Mediterranean center of origin of the genus *Trifolium*. *Ps*-Afghan and *Ps*-Euro are wild (Afghan) and cultivated (European) lines of pea (*P. sativum*). The white oval represents the hypothetical common ancestor (CA) of the species *Rhizobium leguminosarum*, a symbiont of the common ancestor of the IRLC group of legumes of the halegoid complex; in the light gray oval markes ancestral forms of bv. *viciae* with the *nod*X gene (symbionts of *Vavilovia formosa* Steven Fed. and *P. elatius* Bieb. Of the genomic group A) and bv. *trifolii*; in the dark gray oval are evolutionarily advanced forms of bv. *viciae* (most *Ps*-Euro and *Vicia* symbionts belong to genomic group D, which is characterized by the loss of the *nod*X gene). The most probable directions of evolution are shown by solid arrows.

The data obtained allowed us to propose a hypothetical scheme for the evolution of the species *R. leguminosarum* (Fig. 3), according to which its common ancestor (protosymbiont) was able to form symbiosis with ancestral representatives of the IRLC group of legumes of the galegoid complex. The evolution of protosymbionts may have begun with divergence based on host specificity, which

led to the emergence of biovars *viciae* and *trifolii* capable of N_2-fixing symbiosis with plants of only one tribe — *Fabeae* or *Trifolieae*.

In this case, the bv. *trifolii* could arise either directly from the protosymbiont, or through a change in the host specificity of ancestral strains of bv. *viciae*. The second mode is supported by the fact that the distribution area of one of the wild pea species (*P. elatius*) overlaps with the Mediterranean center of origin of the genus *Trifolium* [34, 35], and most strains of bv. *viciae* and bv. *trifolii* exhibit the ability to cross-inoculate with the formation of non-N_2-fixing nodules. It can be assumed that bv. *viciae*, which appears to have evolved faster than bv. *trifolii* due to wider taxonomic diversification of the corresponding host plants, with specialization to newly emerging legume species, the ability to form nodules on clover was lost or replaced by the formation of tumor-like structures in which bacterial infection of plant tissues was impaired.

The data obtained suggest that the symbiotic properties of the common ancestor bv. *viciae* were most preserved by strains isolated from *V. formosa*, 91.6% of which were able to form morphologically normal (Fix^-) nodules on clover, while among strains from vetch and pea only 57.1% showed this ability. In addition, only 41.7% of strains from Vavilovia exhibited the Fix^+ phenotype on vetch, which is shown for all strains from vetch and pea. Hence, strains from Vavilovia which to the greatest extent retained broad host specificity towards different tribes of legumes, have not yet fully acquired the ability to symbiose with evolutionarily young representatives of the tribe *Fabeae*. This confirms the expediency of the previously expressed [4] proposal to assign Vavilovia strains into a separate symbiovar included in bv. *viciae*.

Thus, when studying the host specificity of the species *Rhizobium leguminosarum*, we showed cross-inoculation of biovars *viciae* and *trifolii*, which leads to the formation of nodules that do not fix N_2, having either a normal or an abnormal (tumor-like) structure, and in the second case, infection of plant tissues by bacteria is blocked. Based on the data obtained, a hypothetical scheme of the evolution of the species *R. leguminosarum* was constructed and for the first time a hypothesis is proposed about the emergence of the biovar *trifolii* (which retaines primitive features of the genome organization of this species) through changes in host specificity of ancestral bv. *viciae* strains. The ancestral strains of the bv. *viciae* isolated from the nodules of the relict legume Vavilovia exhibit the highest cross-inoculation activity with clover, indicating that broad host specificity, which in rhizobia is usually combined with low N_2-fixing activity, may be a property that significantly limits the agronomic potential of production strains. Since a significant number of ancestral genetic elements were previously identified in symbionts of the evolutionarily advanced legume tribe *Fabeae*, it is relevant to identiy these elements in the genomes of economically valuable strains. The presence of the *fix*W gene which prevents the full development of bacteroids, limiting their N_2-fixing activity, requires special attention. Since changes in the organization of *sym* genes that occurred during the transition from the ancestral genomic group A to the evolutionarily advanced group D are associated with an increase in the symbiotic activity of bacteria, it is obvious that introducing similar changes into the genomes of commercial strains is of great biotechnological interest. It is obvious that targeted modifications of the genomes of practically valuable *R. leguminosarum* bv. *viciae* strains by editing ancestral genomic elements will help improve the effectiveness of biologicals based on these bacteria.

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